Mitochondrial Gene Cytochrome *b* Developmental and Environmental Expression in *Aedes aegypti* (Diptera: Culicidae)

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ABSTRACT Cytochrome *b*, coded by mitochondrial DNA, is one of the cytochromes involved in electron transport in the respiratory chain of mitochondria. Cytochrome *b* is a critical intermediate in a mitochondrial death pathway. To reveal whether cytochrome *b* of the mosquito *Aedes aegypti* (L.) (Diptera: Culicidae) (*AeaCytB*) is developmentally regulated, we used real-time quantitative polymerase chain reaction (qPCR) to examine *AeaCytB* gene expression levels in different developmental stages of *Ae. aegypti*. The qPCR showed that *AeaCytB* was expressed in each developmental stage, with peaks at first and second instars and was highly expressed in teneral male and female *Ae. aegypti* adults. Because mitochondrial genes exist as multiple copies, *AeaCytB* has much higher expression levels in all developmental stages in *Ae. aegypti* compared with nuclear genes. We also investigated the effect of abiotic environmental factors (e.g., high temperatures, ultraviolet radiation, and pesticide) on *AeaCytB* gene expression. Taken together, these results suggest that *AeaCytB* gene plays an important role in the development of *Ae. aegypti* and its response to environmental stress.

KEY WORDS cytochrome *b*, *Aedes aegypti*, development, quantitative polymerase chain reaction, permethrin

Cytochrome *b*, the cytochrome coded by mitochondrial DNA, is one of the cytochromes involved in electron transport in the mitochondrial respiratory chain (Esposti et al. 1993). Cytochrome *b* is the largest polypeptide in the cytochrome bc_1 complex, which catalyzes the redox transfer of electrons from ubiquinone to cytochrome *c* (Esposti et al. 1993, Crofts et al. 1999). Cytochrome *b* also contains various inhibitors and quinone antagonists that bind and inhibit the activity of oxidoreductase (Esposti et al. 1993). In all eukaryotic and some prokaryotic respiratory chains, energy is obtained from the transfer of electrons through multisubunit complexes (membrane-bound) to cytochrome *c* oxidase (CcO) (Zhen et al. 1999). By reducing oxygen to water, cytochrome *c* provides the electron sink and is the electron conduit between complex III (cytochrome bc_1) and complex IV (CcO) (Zhang et al. 1998, Tian et al. 1999). To provide electrons rapidly, cytochrome *c* must interact with several proteins at a high rate of speed and specificity in the mitochondrial intermembrane space (Zhen et al. 1999).

The role of cytochrome *b* (*AeaCytB*) during development in the mosquito *Aedes aegypti* (L.) (Diptera: Culicidae), a primary vector of dengue and yellow fever viruses, has not been explored. By using real-time quantitative polymerase chain reaction (qPCR), the current study examines the role of the *AeaCytB* gene in developmental expression, environmental response, and pesticide sensitivity in *Ae. aegypti.* Using RNA interference technology to knock down the mitochondrial proteins may provide additional targets that can be developed as new pesticides.

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Materials and Methods

Mosquito Strains. *Ae. aegypti* (Orlando, FL, strain, maintained since 1952) were reared in the insectary of the Mosquito and Fly Research Unit at the Center for Medical, Agricultural, and Veterinary Entomology, USDA–ARS, in Gainesville, FL. Different developmental stages (freshly oviposited eggs, larvae, pupae, and adults) were used for the experiments. Adult females used in theses experiments were not blood fed but were given sucrose as a carbohydrate source for routine maintenance (Pridgeon et al. 2007).

RNA Extraction. All developmental stages of *Ae. aegypti* were collected at several time points within each stage (sample sizes of eggs, larvae, and pupae were 50–100 μg; adults, 20 individuals). Total RNAs were extracted using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Poly(A)$^+$ RNA was isolated with Oligotex-dT (QIAGEN, Valencia, CA). RNA samples were quantified by SmartSpec Plus spectrophotometer (Bio-Rad Laboratories, Hercules, CA).

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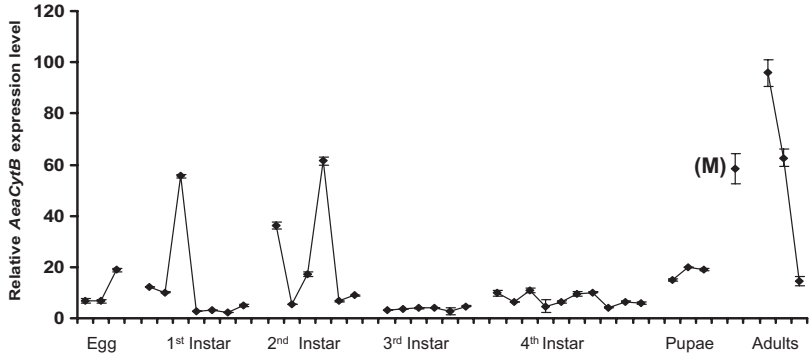

Fig. 1. *AeaCytB* gene expression levels in eggs, larvae, pupae, and adults quantified by qPCR, with SD for three replicates. Ages of eggs, 1, 3, and 6 d, respectively; first instar, 7, 10, 23, 30, 33, 36, and 39 h posthatch, respectively; second instar, 48, 51, 54, 57, 60, and 63 h posthatch, respectively; third instar, 72, 75, 78, 81, 84, and 87 h posthatch, respectively; fourth instar, 96, 99, 102, 105, 108, 111, 129, 132, 145, and 148 h posthatch, respectively; pupae, 154, 157, and 169 h posthatch, respectively; and adults, 1-d-old male, (M) designated male, (i.e.,12 d posthatch); 1-d-old female; 7-d-old female (i.e.,19 d-posthatch); and 14-d-old female (i.e., 26 d posthatch).

Permethrin Experiments. Adult female (3- and 10-d-old) *Ae. aegypti* were treated topically with permethrin/acetone at 2.5×10^{-5} μg (high dose, HD) and 1.25×10^{-5} μg (low dose, LD) per mosquito as described by Pridgeon et al. (2007). Clear 104-ml plastic cups (no. TK35, Solo Sup Company, Highland Park, IL) were used for all treatments in the experiments, including permethrin treatment (15 mosquitos per cup). Fifteen females were collected at 0 (blank control), 5, 15, 30, 60, and 180 min and 6 h after permethrin treatment. A control group was exposed to acetone only (Pridgeon et al. 2007). Mosquito knockdown was recorded for each collection time point and at 24 h postpermethrin treatment (Supplemental Table 5).

Heat-Shock Experiments. *Ae. aegypti* females (2 and 9 d old) were exposed to three temperatures (24, 37, and 40°C) and 56 ± 1.5% RH in an environmental chamber (L-C incubator, Lab-Line Instruments, Inc., Melrose Park, IL) for the time course study. Thirty individuals were collected at 0, 15, 30, and 180 min after heat-shock treatments.

UV-Radiation Experiments. For the UV-radiation time course study, *Ae. aegypti* females (3, 10, and 17 d old) were exposed to a germicidal lamp (30 W, G30TB, General Electric, Fairfield, CT) at the light intensity 1000 μW/cm^2. Thirty individuals were collected at each time point of 0, 15, 30, 180, and 240 min of continuous UV-radiation treatment.

cDNA Synthesis. A 5-μg aliquot of purified RNA was reverse transcribed in a 20-μl reaction volume by using cloned avian myeloblastosis virus first-strand cDNA synthesis system (Invitrogen) for reverse transcription (RT)-PCR according to the manufacturer's instructions. The reaction was terminated after 5 min by heat inactivation at 95°C. The reactions were diluted by adding 80 μl of double distilled H_2O. The cDNA samples for each developmental stage and different experiments were stored at −20°C.

Design of Gene-Specific Primers for RT-PCR. To design gene-specific primers, a detailed analysis of the nucleotide sequence of the mitochondrial gene *AeaCytB* (NCBI accession DQ440235.1) was performed using PRIMER3-Design Primer Pairs and Probes program from Biology Workbench (http://workbench.sdsc.edu). The primers for *Ae. aegypti* actin gene (NCBI accession DQ440059) also were used for an internal control and comparison.

Real-Time PCR Amplification. The qPCR assay for cytochrome *b* gene expression in *Ae. aegypti* (*AeaCytB*) used Platinum SYBR Green qPCR SuperMix-UDG with ROX (Invitrogen) in a volume of 15 μl on a 7300 Fast Real-Time PCR system (Applied Biosystems, Foster City, CA). The PCR mixture consisted of 1 μl of diluted cDNA, 0.5 μM primers, and 1× master mix. In every RT-PCR run, *Actin* was used as an internal control to normalize variation in the amount of cDNA template. The PCR primers used were AEA-CYTB-755F (5′-CTCCTGTCCATATTCAACCAGA-3′) and AEA-CYTB-1004R (5′-TAAGGATCTTCAA-CAGGGCG-3′). The PCR primers for *Actin* are Actin-152F (5′-AGGACTCGTACGTCGGTGAC-3′) and Actin-590R (5′-CGTTCAGTCAGGATCTTC-3′). The PCR thermal cycling parameters were 50°C for 2 min, 95°C for 10 min followed by 40 cycle of 95°C for 15 s and 60°C for 1min. This was followed by the dissociation stage at 95°C for 15 s, 60°C for 30 s, and 95°C for 15 s. Data analysis was based on three replicates of RT-PCR. Relative expression levels were calculated as follows: first, *AeaCytB* transcript levels relative to a standard (*Actin*) by using the formula $\Delta C_T = C_T$ (*AeaCytB*) $- C_T$ (*Actin*). Second, an average ΔC_T value for each sample was calculated. Third, relative expression levels were calculated using the modified equation $1 \times 2^{-[\text{average } \Delta CT]}$ (Portereiko et al. 2006). Because the *AeaCytB* transcript levels were relatively high (≈100 times higher than the nuclear genes), we modified the equation (substituting $1 \times 2^{-[\text{average } \Delta CT]}$ for $100 \times 2^{-[\text{average } \Delta CT]}$).

Statistical Analysis. Comparisons of means were analyzed using Student's *t*-test (Steel et al. 1998), and *t* values and *P* values were reported when nor-

Table 1. Expression of *AeaCytB* in different developmental stages of *Ae. aegyptii*

Sample stage	Sample name	Sample time	Cycle threshold (Ct) ± SD		Relative *AeaCytB* expression level			
			Actin	AeaCytB	ΔCt-1	ΔCt-2	ΔCt-3	$2^{-\Delta Ct}$ ± SD
Egg	Egg 1	1 d	17.841 ± 0.135	15.049 ± 0.055	−2.999	−2.723	−2.654	6.963 ± 0.906
	Egg 2	3 d	17.892 ± 0.106	15.178 ± 0.034	−2.867	−2.596	−2.679	6.582 ± 0.643
	Egg 3	6 d	19.983 ± 0.053	15.744 ± 0.016	−4.292	−4.227	−4.199	18.89 ± 0.626
First-instar larvae	Larvae 1	6 h[a]	17.455 ± 0.050	13.843 ± 0.025	−3.631	−3.584	−3.621	12.23 ± 0.211
	Larvae 2	9 h[a]	17.535 ± 0.019	14.191 ± 0.012	−3.344	−3.359	−3.342	10.14 ± 0.106
	Larvae 3	23 h[a]	20.880 ± 0.040	15.085 ± 0.020	−5.817	−5.778	−5.789	55.51 ± 0.770
	Larvae 4	30 h[a]	16.422 ± 0.015	14.901 ± 0.029	−1.509	−1.552	−1.505	2.871 ± 0.053
	Larvae 5	33 h[a]	16.148 ± 0.046	14.525 ± 0.020	−1.641	−1.593	−1.623	3.080 ± 0.056
	Larvae 6	36 h[a]	15.948 ± 0.029	14.710 ± 0.014	−1.236	−1.255	−1.223	2.359 ± 0.027
	Larvae 7	39 h[a]	16.706 ± 0.090	14.385 ± 0.022	−2.249	−2.448	−2.267	5.007 ± 0.388
Second-instar larvae	Larvae 8	48 h[a]	19.969 ± 0.063	14.790 ± 0.036	−5.220	−5.127	−5.191	36.24 ± 1.189
	Larvae 9	51 h[a]	16.813 ± 0.020	14.336 ± 0.014	−2.458	−2.498	−2.478	5.571 ± 0.078
	Larvae 10	54 h[a]	18.312 ± 0.089	14.206 ± 0.025	−4.140	−4.030	−4.148	17.23 ± 0.776
	Larvae 11	57 h[a]	21.063 ± 0.034	15.121 ± 0.069	−5.917	−5.986	−5.924	61.50 ± 1.647
	Larvae 12	60 h[a]	17.415 ± 0.017	14.696 ± 0.063	−2.720	−2.651	−2.760	6.593 ± 0.273
	Larvae 13	63 h[a]	17.654 ± 0.034	14.487 ± 0.011	−3.145	−3.199	−3.162	8.993 ± 0.171
Third-instar larvae	Larvae 14	72 h[a]	16.194 ± 0.019	14.556 ± 0.013	−1.649	−1.601	−1.655	3.112 ± 0.054
	Larvae 15	75 h[a]	16.929 ± 0.034	15.051 ± 0.009	−1.880	−1.849	−1.904	3.675 ± 0.069
	Larvae 16	78 h[a]	16.894 ± 0.094	14.913 ± 0.019	−2.019	−1.890	−2.033	3.951 ± 0.212
	Larvae 17	81 h[a]	17.079 ± 0.043	15.120 ± 0.032	−1.963	−1.946	−1.970	3.890 ± 0.033
	Larvae 18	84 h[a]	17.215 ± 0.044	15.885 ± 0.878	−1.811	−0.364	−1.815	2.772 ± 1.285
	Larvae 19	87 h[a]	17.333 ± 0.060	15.143 ± 0.021	−2.227	−2.101	−2.240	4.565 ± 0.238
Fourth-instar larvae	Larvae 20	99 h[a]	18.390 ± 0.094	15.103 ± 0.046	−3.200	−3.445	−3.212	9.785 ± 0.961
	Larvae 21	105 h[a]	18.003 ± 0.025	15.316 ± 0.044	−2.674	−2.712	−2.678	6.445 ± 0.095
	Larvae 22	111 h[a]	18.703 ± 0.077	15.256 ± 0.188	−3.374	−3.578	−3.389	10.92 ± 0.881
	Larvae 23	129 h[a]	18.889 ± 0.072	16.779 ± 0.756	−1.707	−2.904	−1.707	4.681 ± 2.429
	Larvae 24	132 h[a]	18.517 ± 0.071	15.829 ± 0.020	−2.732	−2.597	−2.74	6.453 ± 0.348
	Larvae 25	145 h[a]	18.859 ± 0.106	15.627 ± 0.035	−3.302	−3.076	−3.318	9.423 ± 0.860
	Larvae 26	148 h[a]	19.498 ± 0.020	16.158 ± 0.042	−3.133	−3.380	−3.326	10.128 ± 0.251
Pupae	P1	154 h[a]	18.573 ± 0.056	14.683 ± 0.011	−3.914	−3.827	−3.932	14.843 ± 0.574
	P2	157 h[a]	19.009 ± 0.015	14.711 ± 0.015	−4.303	−4.313	−4.308	19.814 ± 0.068
	P3	169 h[a]	18.832 ± 0.045	14.588 ± 0.010	−4.254	−4.200	−4.278	18.953 ± 0.520
Adult	A1 (M)	1 d	21.767 ± 0.081	15.899 ± 0.063	−5.749	−5.829	−6.026	58.587 ± 5.882
	A1 (F)	1 d	21.871 ± 0.107	15.290 ± 0.035	−6.651	−6.493	−6.598	95.818 ± 5.309
	A2 (F)	7 d	19.346 ± 0.282	13.386 ± 0.080	−6.072	−5.726	−6.077	62.648 ± 3.431
	A3 (F)	14 d	19.288 ± 0.164	15.435 ± 0.040	−3.959	−3.656	−3.943	14.508 ± 1.648

[a] Hours posthatch.

mality and equal variance tests were passed. If a *t*-test failed, the data were analyzed by the Mann–Whitney rank sum test (SigmaStat 3.5, Systat Software, Inc. San Jose, CA).

Results

***AeaCytB* Gene Regulation in Different Developmental Stages of *Ae. aegypti*.** To understand how mitochondrial genes are regulated during the development of *Ae. aegypti*, we examined cytochrome *b* (*AeaCytB*) relative expression levels in eggs, larvae, pupae, and adults by using qPCR (Fig. 1).

In the egg stage, the relative RNA expression level of *AeaCytB* increased over time from day 1 to day 6. About halfway through the development of the first-instar larva (23 h posthatch), the RNA relative expression level of *AeaCytB* was slightly higher than in the early (7 and 10 h posthatch) and late (39 h posthatch) stage samples. *AeaCytB* expression was relatively high in the early second-instar larvae (48 h posthatch) and the middle-late second-instar larvae (57 h posthatch). However, the RNA expression level of *AeaCytB* was relatively low in the third- and fourth-instar larvae examined (from 72 to 148 h posthatch). For the pupal stage, RNA relative expression level of *AeaCytB* increased slightly over time (from 14.84 ± 0.57 to 18.95 ± 0.52) (Table 1). However, RNA relative expression level of *AeaCytB* increased significantly ($P = 0.001$) between teneral male (58.59 ± 5.88) and female (95.82 ± 5.31) mosquitoes. RNA expression of *AeaCytB* in teneral female *Ae. aegypti* was significant higher than that found in 7- and 14-d-old adults (Table 1; Supplemental Table 1A). It is interesting that expression of *AeaCytB* in male mosquitoes (>7 d old) was not detected.

Effects of Permethrin on *AeaCytB* Expression in Adult *Ae. aegypti*. To determine whether the expression of *AeaCytB* gene in *Ae. aegypti* was affected by permethrin treatment, mosquitoes of different ages were treated with different concentrations of permethrin as described previously using acetone as a

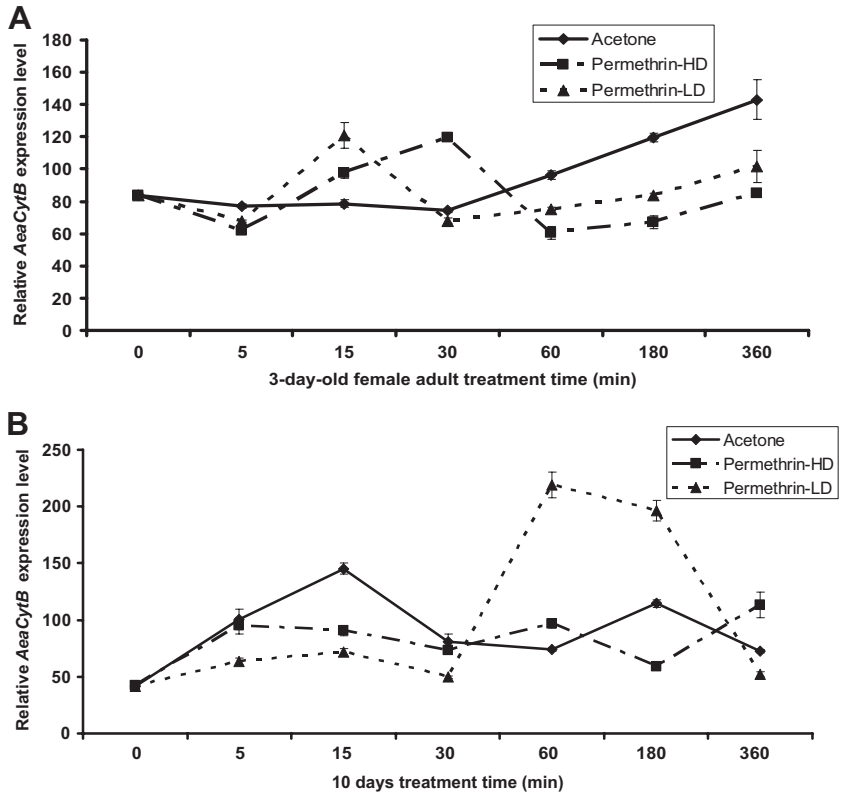

Fig. 2. (A and B) *AeaCytB* gene expression levels in 3- and 10-d-old adults treated topically with permethrin/acetone at HD and LD, quantified by qPCR, with SD for three replicates. Please note x-axis is not to scale. (A) Three-day-old adults postexposure to permethrin at 0, 5, 15, 30, 60, 180, and 360 min. (B) Ten-day-old adults postexposure permethrin at 0, 5, 15, 30, 60, 180, and 360 min.

carrier (Pridgeon et al. 2007). The qPCR time courses of *AeaCytB* expressed in 3- and 10-d-old female *Ae. aegypti* were different between two concentrations of the permethrin (HD and LD). In 3-d-old female *Ae. aegypti*, *AeaCytB* expression decreased slightly for both doses 5 min postpermethrin treatment (61.76 ± 1.78 [LD] and 67.52 ± 0.67 [HD]) and then increased at 15 min posttreatment (97.62 ± 3.12 [LD] and 120.97 ± 8.12 [HD]) compared with treatment with acetone only as a control (78.68 ± 2.39) (Fig. 2A; Table 2). For permethrin/acetone HD-treated *Ae. aegypti* adults continued to show an increase in *AeaCytB* gene expression (119.51 ± 1.45) at 30 min postexposure, but permethrin/acetone LD-treated *Ae. aegypti* adults decrease in *AeaCytB* gene expression (67.88 ± 1.75) at 30 min postexposure compared with treatment with acetone only (74.13 ± 1.24) as a control (Fig. 2A; Table 2). In 3-d-old *Ae. aegypti* adults, *AeaCytB* expression at permethrin/acetone LD-treated *Ae. aegypti* adults decreased significantly as postpermethrin treatment time increased from 60 to 180 to 360 min compared with treatment with acetone only as a control (Fig. 2A; Supplemental Table 2A).

The qPCR data showed that *AeaCytB* expression levels were up-regulated in 10-d-old adult *Ae. aegypti* treated topically either with permethrin/acetone (HD and LD) or with acetone alone (Fig. 2B). In 10-d-old females, *AeaCytB* expression after 5, 15, and 30 min posttreatment with permethrin was less than acetone treatment alone (Fig. 2B). However, after 60 min posttreatment with permethrin/acetone LD, *AeaCytB* expression was significantly greater (219.14 ± 11.36) than acetone treatments (74.15 ± 1.51) ($P < 0.001$) (Supplemental Table 2B). Permethrin/acetone LD treated *Ae. aegypti* adults continued to show a significant increase in *AeaCytB* gene expression until 3 h postexposure (Fig. 2B; Supplemental Table 2B). This result demonstrated that *AeaCytB* expression levels differ by age in adult mosquitoes in response to permethrin treatment.

Effects of Heat Stress and UV-Radiation on *AeaCytB* Gene Expression in Female *Ae. aegypti*. To understand the effects of extreme environmental conditions on mosquito survival and cytochrome *b* regulation in nature, we conducted heat shock and UV radiation experiments on adults of different ages and examined *AeaCytB* gene expression levels. RT-PCR data indicated that *AeaCytB* gene expression increased after 15 min of heat exposure in both young (2-d-old) and older (9-d-old) adults at 37 and 40°C, respectively (Fig. 3; Table 3). However, after

Table 2. Expression of *AeaCytB* in acetone and permethrin/acetone treatments in 3- and 10-d-old female adult *Ae. aegypti*

Sample name	Cycle threshold (Ct) ± SD		Relative *AeaCytB* expression level			
	Actin	AeaCytB	ΔCt-1	ΔCt-2	ΔCt-3	$2^{-\Delta Ct}$ ± SD
A3d[a] — 0′	17.600 ± 0.060	11.208 ± 0.068	−6.373	−6.418	−6.383	83.956 ± 1.376
A3d[a] — 5′	17.427 ± 0.038	11.156 ± 0.044	−6.247	−6.298	−6.267	77.216 ± 1.389
A3d[a] — 15′	17.479 ± 0.032	11.182 ± 0.013	−6.348	−6.275	−6.270	78.683 ± 2.394
A3d[a] — 30′	17.264 ± 0.008	11.052 ± 0.019	−6.209	−6.189	−6.237	74.126 ± 1.243
A3d[a] — 60′	17.444 ± 0.025	10.853 ± 0.029	−6.551	−6.624	−6.600	96.449 ± 2.492
A3d[a] — 180′	17.899 ± 0.042	10.998 ± 0.010	−6.920	−6.921	−6.863	119.55 ± 2.728
A3d[a] — 360′	18.331 ± 0.163	11.173 ± 0.041	−7.288	−7.143	−7.041	143.11 ± 12.39
P[1]-3d[b] — 0′	17.600 ± 0.060	11.208 ± 0.068	−6.373	−6.418	−6.383	83.956 ± 1.376
P[1]-3d[b] — 0′	17.139 ± 0.011	11.191 ± 0.030	−5.995	−6.658	−6.600	61.758 ± 1.782
P[1]-3d[b] — 15′	17.942 ± 0.026	11.334 ± 0.020	−6.658	−6.600	−6.568	97.617 ± 3.115
P[1]-3d[b] — 30′	18.082 ± 0.020	11.184 ± 0.038	−6.919	−6.885	−6.898	119.51 ± 1.447
P[1]-3d[b] — 60′	16.954 ± 0.049	11.038 ± 0.063	−5.821	−5.918	−6.009	60.463 ± 3.924
P[1]-3d[b] — 180′	17.249 ± 0.106	11.184 ± 0.046	−6.052	−5.990	−6.154	67.038 ± 3.959
P[1]-3d[b] — 360′	17.622 ± 0.023	11.209 ± 0.019	−6.401	−6.410	−6.426	85.174 ± 0.769
P[2]-3d[c] — 0′	17.600 ± 0.060	11.208 ± 0.068	−6.373	−6.418	−6.383	83.956 ± 1.376
P[2]-3d[c] — 5′	17.373 ± 0.010	11.296 ± 0.014	−6.062	−6.091	−6.078	67.517 ± 0.669
P[2]-3d[c] — 15′	18.063 ± 0.104	11.147 ± 0.007	−7.013	−6.916	−6.819	120.97 ± 8.121
P[2]-3d[c] — 30′	17.188 ± 0.049	11.104 ± 0.018	−6.097	−6.114	−6.043	67.879 ± 1.745
P[2]-3d[c] — 60′	17.334 ± 0.032	11.099 ± 0.015	−6.249	−6.239	−6.216	75.326 ± 0.888
P[2]-3d[c] — 180′	17.499 ± 0.029	11.113 ± 0.024	−6.367	−6.383	−6.407	83.623 ± 1.164
P[2]-3d[c] — 360′	17.954 ± 0.183	11.290 ± 0.045	−6.522	−6.670	−6.799	101.69 ± 9.733
A10d[d] — 0′	15.112 ± 0.012	9.738 ± 0.064	−5.312	−5.456	−5.471	41.461 ± 2.220
A10d[d] — 5′	16.267 ± 0.117	9.624 ± 0.014	−6.513	−6.643	−6.774	100.25 ± 9.071
A10d[d] — 15′	16.777 ± 0.065	9.596 ± 0.016	−7.124	−7.218	−7.202	145.20 ± 5.030
A10d[d] — 30′	15.768 ± 0.054	9.428 ± 0.056	−6.231	−6.341	−6.450	81.191 ± 6.178
A10d[d] — 60′	15.590 ± 0.030	9.378 ± 0.005	−6.226	−6.232	−6.178	74.146 ± 1.514
A10d[d] — 180′	16.398 ± 0.045	9.559 ± 0.006	−6.798	−6.843	−6.876	114.49 ± 3.119
A10d[d] — 360′	15.899 ± 0.017	9.716 ± 0.018	−6.204	−6.195	−6.149	72.650 ± 1.475
P[1]-10d[e] — 0′	15.108 ± 0.053	9.715 ± 0.148	−5.470	−5.427	−5.280	42.070 ± 2.863
P[1]-10d[e] — 5′	16.151 ± 0.075	9.585 ± 0.038	−6.437	−6.622	−6.641	94.986 ± 7.267
P[1]-10d[e] — 15′	16.138 ± 0.069	9.636 ± 0.074	−6.452	−6.589	−6.467	90.753 ± 4.769
P[1]-10d[e] — 30′	15.751 ± 0.032	9.556 ± 0.016	−6.166	−6.220	−6.198	73.247 ± 1.381
P[1]-10d[e] — 60′	16.233 ± 0.014	9.635 ± 0.044	−6.665	−6.557	−6.573	96.938 ± 3.941
P[1]-10d[e] — 180′	15.326 ± 0.062	9.439 ± 0.079	−5.864	−5.925	−5.873	59.205 ± 1.353
P[1]-10d[e] — 360′	16.361 ± 0.152	9.540 ± 0.010	−6.912	−6.900	−6.651	113.44 ± 11.25
P[2]-10d[f] — 0′	15.111 ± 0.012	9.739 ± 0.064	−5.348	−5.457	−5.311	41.451 ± 2.208
P[2]-10d[f] — 5′	15.635 ± 0.099	9.646 ± 0.137	−5.971	−6.066	−5.931	63.579 ± 3.097
P[2]-10d[f] — 15′	15.653 ± 0.062	9.487 ± 0.002	−6.101	−6.178	−6.219	71.842 ± 2.946
P[2]-10d[f] — 30′	15.257 ± 0.030	9.623 ± 0.012	−5.672	−5.633	−5.597	49.664 ± 1.298
P[2]-10d[f] — 60′	17.458 ± 0.185	9.684 ± 0.120	−7.814	−7.822	−7.687	219.14 ± 11.36
P[2]-10d[f] — 180′	17.190 ± 0.048	9.575 ± 0.023	−7.687	−7.601	−7.557	196.17 ± 9.067
P[2]-10d[f] — 360′	15.812 ± 0.092	10.113 ± 0.024	−5.711	−5.761	−5.624	51.986 ± 2.487

[a] Acetone treatments in 3-d-old female adult *Ae. aegypti.*
[b] Permethrin HD treatments in 3-d-old female adult *Ae. aegypti.*
[c] Permethrin LD treatments in 3-d-old female adult *Ae. aegypti.*
[d] Acetone treatments in 10-d-old female adult *Ae. aegypti.*
[e] Permethrin HD treatments in 10-d-old female adult *Ae. aegypti.*
[f] Permethrin LD treatments in 10-d-old female adult *Ae. aegypti.*

30 or 180 min of heat exposure at 40°C, *AeaCytB* gene expression was reduced in 2-d-old adults to levels present in the controls (Fig. 3A; Table 3). In addition, different temperatures showed age-related effects on *AeaCytB* gene expression. Two-day-old females had higher *AeaCytB* gene expression at 40°C than at 24°C after 15 min of heat exposure. In contrast, the 9-d-old females had higher *AeaCytB* gene expression at 37°C than at 24°C after 15 min of heat exposure. This indicates that environmental temperatures affect *AeaCytB* gene expression and have the greatest effect when first induced.

Using RT-PCR, we examined expression of the *AeaCytB* in *Ae. aegypti* after treatment with UV-radiation. *AeaCytB* gene expression increased significantly ($P < 0.001$) in 3-d-old adults after 15 min of UV light exposure (Fig. 4; Table 4). For 3-d-old females, relative *AeaCytB* gene expression levels after UV exposure increased from 0 min (37.3 ± 0.39) to 15 min (263.4 ± 2.5) but decreased after 30 min (57.9 ± 2.5). After 3 and 6 h of UV exposure, the relative *AeaCytB* gene expression levels slightly decreased (28.96 ± 1.8 and 41.1 ± 0.04, respectively). Statistical comparisons of relative *AeaCytB* expression between the different UV exposure times in 3-d-old adult *Ae. aegypti* indicated significant differences ($P < 0.001$) between 0- and 15-min and 15- and 30-min UV exposure (Supplemental Table 4A). However, the relative *AeaCytB* expression level in 10- and 17-d-old adults changed very little after UV exposure (Fig. 4). These data show that various ages of *Ae. aegypti* adults have different sensitivities to UV exposure as measured by the regulation of *AeaCytB.*

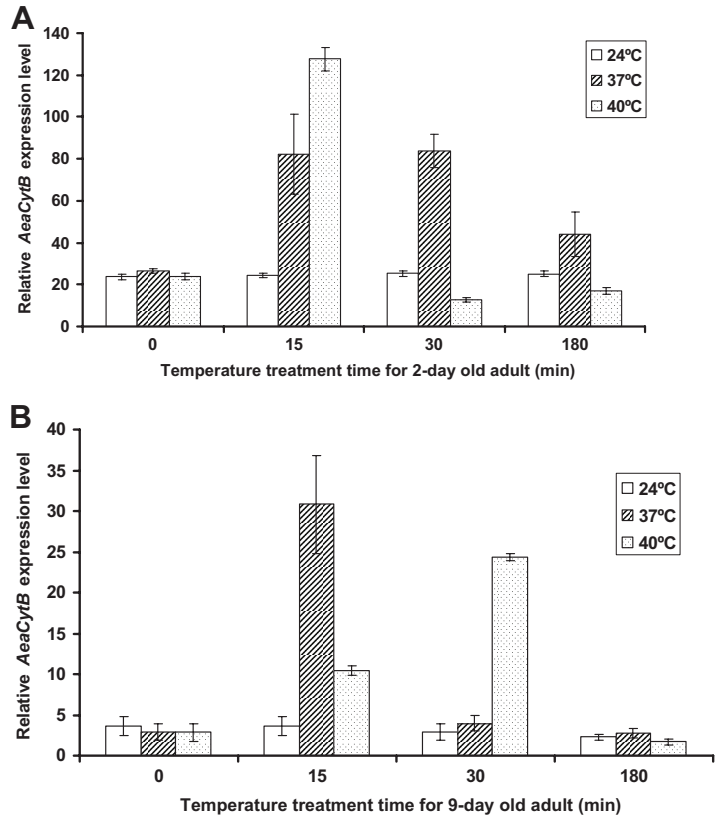

Fig. 3. (A and B) *AeaCytB* gene expression levels in 2- and 9-d-old adults exposed to different temperatures, quantified by qPCR, with SD for three replicates. Please note x-axis is not to scale. (A) Two-day-old adults exposed at 24, 37, and 40°C. (B) Nine-day-old adults exposed at 24, 37, and 40°C.

Discussion

Mitochondrial Gene Cytochrome *b* Expression during *Ae. aegypti* Development. We analyzed changes in the mitochondrial gene expression of cytochrome *b* in *Ae. aegypti* eggs, larvae, pupae, and adults. The mitochondria gene *AeaCytB* is expressed >100-fold higher than nuclear genes, such as *AeaCytC*, during all developmental stages examined (Zhao et al. 2008). The *AeaCytB* gene is expressed at relatively low levels in the late larval and pupal stages and is expressed in varying quantities during the egg stages and early larval instars. In adults, *AeaCytB* expression differed in males and females of different ages. In females, *AeaCytB* gene expression is higher than in males for all ages examined. There were significant differences in the expression of *AeaCytB* genes between teneral and 14-d-old *Ae. aegypti* females. In addition, *AeaCytB* gene expression was significantly different between teneral males and females. However, the relative RNA levels of *AeaCytB* expression was undetectable in the older male mosquitoes (>7-d-old), which may suggest that *AeaCytB* plays an important and different role depending on adult *Ae. aegypti* sex and age. Numerous physiological changes occur during the development of male and female mosquitoes as they grow older and *AeaCytB* gene expression in mature mosquitoes is critical for mitochondrial functions and may be related to mosquito aging. The relative low levels of *AeaCytB* gene expression in older or senescent males and females suggest that mitochondrial dysfunction may also play a role in the attenuation of gene expression.

Mosquito development can be regulated by multinuclear genes (Severson et al. 2004, Fontenille et al. 2005, Raibaud et al. 2006, Strode et al. 2006, van den Hurk et al. 2007) and also may be regulated by mitochondrial genes. The age-related elevation in adenine nucleotide translocase carbonyl content indicate that proteins in mitochondrial membranes are modified selectively during aging (Yan and Sohal 1998).

The integral membrane protein component of the cytochrome bc_1 complex, which catalyzes the redox transfer of electrons from ubiquinone to cytochrome *c*, and the *AeaCytB* gene expression might correlate to the cytochrome *c* genes expression in the adult mosquitoes (Zhao et al. 2008).

Effect of Permethrin on *AeaCytB* Gene Expression. Mosquitoes have an efficient defense system against pesticide challenge that usually involves metabolic resistance mechanism (Etang et al. 2007). Molecular studies of insecticide resistance, including identification of genes involved in target site and metabolic resistance mechanisms, have advanced rapidly over

Table 3. Expression of *AeaCytB* under heat stress conditions

Sample name	Cycle threshold (Ct) ± SD		Relative *AeaCytB* expression level			
	Actin	AeaCytB	ΔCt-1	ΔCt-2	ΔCt-3	$2^{-\Delta Ct}$ ± SD
24°C2d — 0′	20.888 ± 0.399	15.778 ± 0.320	−4.663	−4.512	−4.536	23.784 ± 1.358
24°C2d — 15′	20.299 ± 0.380	15.701 ± 0.342	−4.529	−4.625	−4.640	24.231 ± 0.998
24°C2d — 30′	20.355 ± 0.412	15.699 ± 0.343	−4.594	−4.637	−4.736	25.226 ± 1.278
24°C2d — 180′	20.295 ± 0.379	15.702 ± 0.341	−4.594	−4.735	−4.625	25.156 ± 1.312
37°C2d — 0′	20.298 ± 0.425	15.787 ± 0.321	−4.670	−4.780	−4.735	26.525 ± 1.008
37°C2d — 15′	23.323 ± 0.381	17.325 ± 0.872	−5.924	−6.598	−6.470	82.093 ± 18.96
37°C2d — 30′	21.797 ± 0.449	15.416 ± 0.316	−6.320	−6.288	−6.535	83.569 ± 7.959
37°C2d — 180′	21.375 ± 0.489	15.946 ± 0.370	−5.568	−5.007	−5.711	43.999 ± 10.55
40°C2d — 0′	20.889 ± 0.340	15.780 ± 0.321	−4.662	−4.510	−4.539	23.780 ± 1.456
40°C2d — 15′	23.571 ± 0.446	16.575 ± 0.386	−6.945	−6.978	−7.064	127.66 ± 5.486
40°C2d — 30′	19.362 ± 0.424	15.682 ± 0.328	−3.656	−3.592	−3.792	12.838 ± 0.919
40°C2d — 180	19.328 ± 0.419	15.240 ± 0.300	−4.020	−4.019	−4.225	17.045 ± 1.432
24°C9d — 0′	18.315 ± 1.222	16.529 ± 0.788	−2.015	−1.194	−2.146	3.587 ± 1.141
24°C9d — 15′	18.549 ± 0.908	16.772 ± 0.604	−2.146	−1.169	−2.015	3.573 ± 1.164
24°C9d — 30′	17.736 ± 0.564	16.276 ± 0.365	−1.169	−2.015	−1.194	2.860 ± 1.025
24°C9d — 180′	17.844 ± 0.721	16.674 ± 0.744	−1.194	−1.147	−1.169	2.250 ± 0.371
37°C9d — 0′	18.216 ± 0.370	16.772 ± 0.604	−1.147	−1.169	−2.014	2.859 ± 1.025
37°C9d — 15′	29.136 ± 0.231	24.207 ± 0.039	−4.774	−4.774	−5.241	30.84 ± 6.038
37°C9d — 30′	23.202 ± 0.931	21.479 ± 0.631	−2.053	−1.552	−2.246	3.9415 ± 0.924
37°C9d — 180′	23.783 ± 0.752	22.366 ± 0.533	−1.555	−1.039	−1.658	2.7166 ± 0.582
40°C9d — 0′	18.216 ± 0.370	16.771 ± 0.604	−2.015	−1.170	−1.150	2.8353 ± 1.046
40°C9d — 15′	26.726 ± 0.659	23.348 ± 0.652	−3.371	−3.378	−3.875	10.403 ± 0.0594
40°C9d — 30′	28.729 ± 0.981	24.124 ± 0.996	−4.593	−4.634	−4.589	24.341 ± 0.416
40°C9d — 180	22.475 ± 0.541	21.692 ± 0.520	−0.797	−0.747	−0.803	1.7200 ± 0.363

the past decade. The evolution of insecticide resistance acts by selection of these mechanisms, typically requiring the interaction of multiple genes (Liu et al. 2007, Zhao et al. 2008). The expression of approximately one quarter of the detoxification genes in *Anopheles gambiae* Giles were found to be developmentally regulated (Strode et al. 2006). Our qPCR data showed that *AeaCytB* expression levels of 3-d-old adult *Ae. aegypti* were down-regulated at 5 min and were up-regulated at 15 min after permethrin treatment, whereas 10-d-old adults treated with permethrin showed that *AeaCytB* expression was down-regulate at both 5 and 15 min. The qPCR data showed that *AeaCytB* expression levels of 3-d-old adults *Ae. aegypti* were up regulated at 60, 180, and 360 min after the acetone treatment alone. One hour posttreatment, 3-d-old control adults showed only slightly higher *AeaCytB* expression levels than that in the permethrin treatments, whereas 10-d-old adults treated with permethrin/acetone LD showed a greater than three-fold increase in *AeaCytB* expression than that in the acetone controls. There were significant differences in the relative levels of *AeaCytB* expression at 60 and 180 min posttreatment between the permethrin/acetone LD and acetone treatments. This indicates that *AeaCytB* gene function in adult mosquitoes may increase with age in response to permethrin treatment.

Cytochrome *b* Responses to Abiotic Environmental Stresses. The *AeaCytB* gene of *Ae. aegypti* responds differently to challenge by heat stress and UV radiation. Temperature affects biochemical, physiological, and behavioral processes in mosquitoes (Narang and Narang 1975, Beach et al. 1989, Mahmood and Crans 1997, Wiwatanaratanabutr and Kittayapong 2006). In this study, the influence of heat stress on the expression of the *AeaCytB* gene was shown to be similar

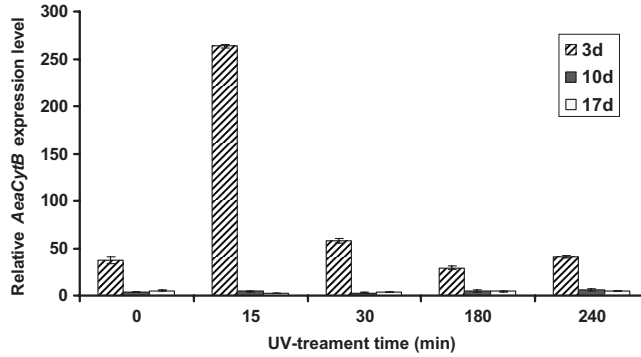

Fig. 4. Time course of *AeaCytB* expression in *Ae. aegypti* adults of different ages after UV light treatment quantified by real-time PCR, with SD for three replicates. Please note x-axis is not to scale. Three, 10-, and 17-d-old mosquitoes were exposed to UV light for 0, 15, 30, 180, and 240 min.

Table 4. Expression of *AeaCytB* under UV stress conditions

Sample name	Cycle threshold (Ct) ± SD		Relative *AeaCytB* expression level			
	Actin	AeaCytB	ΔCt-1	ΔCt-2	ΔCt-3	$2^{-\Delta Ct}$ ± SD
UV-3d — 0 min	19.032 ± 0.228	13.815 ± 0.082	−5.132	−5.386	−5.131	37.319 ± 3.895
UV-3d — 15 min	24.034 ± 0.003	15.993 ± 0.009	−8.048	−8.027	−8.047	263.42 ± 2.050
UV-3d — 30 min	19.233 ± 0.053	13.378 ± 0.009	−5.823	−5.925	−5.816	57.904 ± 2.487
UV-3d — 180 min	17.026 ± 0.053	12.172 ± 0.142	−4.933	−4.872	−4.757	28.955 ± 1.771
UV-3d — 240min	18.834 ± 0.063	13.475 ± 0.064	−5.360	−5.358	−5.361	41.047 ± 0.039
UV-10d — 0 min	18.622 ± 0.072	16.791 ± 0.076	−1.955	−1.871	−1.666	3.5703 ± 0.360
UV-10d — 15 min	23.445 ± 0.174	21.362 ± 0.132	−2.071	−2.131	−2.046	4.2379 ± 0.129
UV-10d — 30 min	15.999 ± 0.209	14.470 ± 0.696	−1.813	−1.809	−0.968	2.9911 ± 0.896
UV-10d — 180 min	17.835 ± 0.039	15.504 ± 0.376	−2.682	−2.378	−1.934	5.1462 ± 1.298
UV-10d — 240 min	16.768 ± 0.112	14.353 ± 0.679	−2.716	−2.570	−1.961	5.5937 ± 1.192
UV-17d — 0 min	32.223 ± 1.718	29.905 ± 1.588	−2.545	−2.162	−2.246	5.0182 ± 0.719
UV-17d — 15 min	17.871 ± 0.022	16.473 ± 0.139	−1.485	−1.478	−1.232	2.6439 ± 0.256
UV-17d — 30 min	16.862 ± 0.015	15.096 ± 0.012	−1.791	−1.796	−1.790	3.4643 ± 0.008
UV-17d — 180 min	17.123 ± 0.048	14.995 ± 0.046	−2.184	−2.018	−2.183	4.3779 ± 0.284
UV-17d — 240 min	17.080 ± 0.002	14.909 ± 0.029	−2.153	−2.208	−2.153	4.5053 ± 0.099

(up-regulated) in 2- and 9-d-old adult mosquitoes. However, *AeaCytB* gene expression was up-regulated at much higher levels in 2-d-old *Ae. aegypti* adults at 40°C than in 9-d-old adults at either 37 or 40°C. After the 15 or 30 min of heat treatment, the up-regulated *AeaCytB* gene may indicate increased respiratory demands for mosquitoes at high temperatures. However, at the optimal temperature (i.e., 24°C), *AeaCytB* gene expression showed little variability after 30 min of heat treatment. The current study clearly demonstrates that mosquitoes respond to heat stress by regulating mitochondrial *AeaCytB* gene expression. As reported previously, human mitochondrial DNA cytochrome *b* gene expression can be detected from mosquitoes after bloodmeal ingestion (Oshaghi et al. 2006). For this reason, we used females that were not blood fed but had sucrose available during the course of the experiments.

UV radiation is known to affect some physiological changes in adult mosquitoes (Beard 1972, Sugumaran et al. 1992, Jayachandran and Fallon 2004). In a previous study on adult *An. gambiae*, there were quantitative differences in 48 cuticular hydrocarbons between young individuals (0–2 d) and older individuals (Caputo et al. 2005). *AeaCytB* gene expression levels in 3-d-old *Ae. aegypti* were significantly increased after 15-min UV treatment and then significantly reduced after 30-min UV treatment (Supplemental Table 4A). However, the *AeaCytB* gene expression level in 10- and 17-d-old *Ae. aegypti* remained largely unchanged. This result may suggest that older mosquitoes have more protection to UV radiation, possibly due to changes in cuticle composition that occurs with age.

In conclusion, *AeaCytB* gene expression in the life cycle of *Ae. aegypti* is highly regulated both developmentally and in response to environment stresses. Cytochrome *b* activity has, for the first time, been examined in detail for all developmental stages of a mosquito. The current study suggests that the mitochondrial gene *AeaCytB* plays an important functional role in *Ae. aegypti* and may provide critical information needed for designing novel control strategies for medically important disease vectors and identifying new pathways to target for the development of new pesticides (Pridgeon et al. 2008).

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